Asian Herpetological Research 2012, 3(4): 322–326
DOI: 10.3724/SP.J.1245.2012.00322

Histological Study of the Liver Pigmentation of Chinese Fire-bellied Newt (*Cynops orientalis*) During Activity and Hibernation Periods

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Abstract The pigmentation in the liver of Chinese fire-bellied newt (*Cynops orientalis*) was described during two periods of the annual cycle (summer activity and winter hibernation). A large number of melanin granules were gathered into clusters and distributed unevenly inside the pigment cells. Liver pigmentation (melanin content) was found unstable, varying during the annual cycle. During the hibernation period, pigmentation accumulation was shown to increase in the liver of the Chinese fire-bellied newt. Hepatocytes during the active period are approximately 14.64% larger than those in the hibernation period, while the nucleus is approximately 7.43% bigger during the active period when compared with that during the hibernation period. These findings indicate that variation in pigment distribution and hepatocyte morphology in Chinese fire-bellied newt liver may be an ecologically adaptive strategy to the adverse physiological conditions during hibernation.

Keywords Chinese fire-bellied newt, histology, pigment cell, hepatocyte, seasonal variation

1. Introduction

Previous studies found that pigment cells of Amphibia as Kupffer cells belong to the "mononuclear phagocyte system" (van Furth *et al*., 1972), and are localized to the wall of the hepatic sinusoid, particularly in the portal area. Pigment cells also occur in the sinusoid walls and have the ability to synthesize pigment (Scalia *et al*., 1988). Based on the localization and phagocytic capacity of the pigment cells, some experts think they are derived from Kupffer cells (Barni *et al*., 1999; Guida *et al*., 2000). Pigment cells occur in various tissues and organs such as liver, skin, spleen and kidney of amphibians and fishes. Moreover, pigmentation changes during different seasons and ages of the newt (Agius and Roberts, 2003; Frangioni *et al*., 2005; Jordanova *et al*., 2008; Slominski *et al*., 2004; Mizell, 1965; Moura *et al*., 2009; Zuasti *et al*., 1989). The pigment cells present in the liver of fishes and amphibians belong to the extracutaneous pigment system (Breathnach, 1988).

As for amphibians, many experimental results suggested that liver pigment cells could synthesize melanin (Barni *et al*., 1999; Pintucci *et al*., 1990; Mizell, 1965). As a consequence of the adaptation to the long period of winter with low environmental temperature and food deprivation, the pigment cells could undergo the drastic changes in structure and function during the natural hibernation (Barni *et al*., 1999, 2002; Frangioni *et al*., 2003, 2005). Moreover, liver pigmentation has been found unstable, with a maximum content during the hibernation period and a minimum content during activity period (Barni *et al*., 1999).

In fishes, pigment cells usually vary in pigmentation quantity, as previous studies documented that the count, size of the pigment cells and the content of pigmentation increased when fishes aged, during the spawning period or with the presence of cachectic disease (Agius, 1979; Agius and Roberts, 2003; Jordanova *et al*., 2008). Additionally, pigment cells evoke responses to environment pollution and climate change. So the pigment cells could also be used as a bio-indicator to better monitor and understand

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Received: 27 March 2012 Accepted: 12 July 2012

the environment and climate changes (Blazer *et al*., 1994; Alcorn *et al*., 2002; Jordanova *et al*., 2008).

Until now, little is known about the morphological and histological aspects of the pigmentation in the liver of Chinese fire-belled newt. The present study aimed to investigate the distinctive morphology of the pigment cells and the changes of the pigmentation in the Chinese fire-bellied newt liver during an annual cycle.

2. Materials and Methods

2.1 Animals used in this experiment In total 68 female and 26 male Chinese fire-bellied newts, *Cynops orientalis* (36 females and 17 males were collected during the hibernation period, with the remaining during the active period), with an average length of 40.1 mm ± 2.0 mm, and average weight of 1.90 g ± 0.33 g. They were captured in ponds, streams and wetlands near Guangshui County, Hubei, China in two successive years during the active period (June, with mean environmental temperature of 28°C), and in the hibernation period (January and February, with mean environmental temperature of 3°C).

2.2 Sampling and analysis of tissues After anesthetization with a lethal dose of sodium pentobarbital, the animals were killed and dissected. The livers were immediately taken for later analysis. For light microscopy, the samples were fixed in 10% neutral buffered formaldehyde and Bouin's solution without acetic acid (3:1 mixture of saturated solution of picric acid in water and formalin) for 48 h. Samples were then dehydrated through a series of graded alcohols, cleared in xylene, infiltrated, and embedded into paraffin. Liver paraffin wax blocks were cut into 7 μm thick sections, stained with hematoxylin and eosin, and then they were examined under Nikon TE2000-U microscope. The measurements were conducted on TE200-U (Nikon) image analysis system in randomly chosen microscope fields (30 for each individual), including both the cells and their nucleus diameter. All the data were expressed graphically as mean values ± SD. Statistical comparisons among the different annual periods were analyzed by one-way ANOVA. A value of $P < 0.05$ was accepted for statistical significance.

For electron microscopy, the tissue blocks were fixed in 2% osmium tetroxide immediately for 2 h at room temperature and then rinsed thoroughly to wash out the OsO_4 solution on their surface. After dehydration through a series of graded alcohols, the tissue blocks were embedded in Epon 812. The ultrathin sections (70–80 nm) were post-stained with 2% uranyl acetate for 30 min, washed with double distilled water, counterstained with 2% lead citrate and washed again. The ultrathin sections should be cut sagittally in the median plane from the superficial part of the liver. Finally, the transmission electron microscope was used to examine the ultrathin tissue slices. All the procedures used in this study concerning the animal treatment were in accordance with the guidelines of Law of China on the Protection of Wildlife (1988).

3. Results

Under light microscopy, it is observed that the newt's pigment cells, also known as Kupffer cells or melanomacrophages, are localized mainly in the wall of the hepatic sinusoids (Figure 1 A, B) and in the haematopoietic subcapsular tissue of the liver (Figure 2 C, F). They are interpolated between the endothelial cells (Figure 1 B, E). At the transmission electron microscope level, the pigment cells are irregular and dendritically shaped, and there are large numbers of melanin granules being gathered into cluster and distributed unevenly (Figure 2 A). Stained with uranyl acetate and lead citrate, they were similar to the nucleolus in appearance. Melanin granules were round in shape, consisting of electron-dense granules, and containing very dense homogenous material shown by a thin peripheral rim, and haphazardly, situated in the cytoplasm without particular relationships of contiguity to the usual cytoplasmic organelles (Figure 2 B). Numerous smooth endoplasmic reticulums with abundant ribosomes were clearly observed in the cytoplasm of the pigment cell when magnified (Figure 2 B).

The data for the newt liver weight (LW), nucleus diameter (ND), cell diameter (CD), and the area of the liver section occupied by the pigmentation which is expressed by S_p/S_t during the two periods of the annual

Table 1 Morphometric characteristics of histomorphology of the liver in *C. orientalis*.

Period	LW (g)	ND (μm)	CD (μm)	V_v (%)	S_p/S_t (%)
Activity	0.28 ± 0.05	4.03 ± 0.39	7.27 ± 1.02	0.24 ± 0.11	0.09 ± 0.03
Hibernation	0.23 ± 0.07	3.44 ± 0.70	6.73 ± 1.05	0.18 ± 0.12	0.16 ± 0.08

LW: Liver weight; ND: Nucleus Diameter; CD: Cell Diameter; V_V: $V_{nucleus}$ / V_{cell}; S_p: The area of the pigmentation; S_t: Total area of the liver; S_p/S_t : Area of the liver section occupied by the pigmentation.

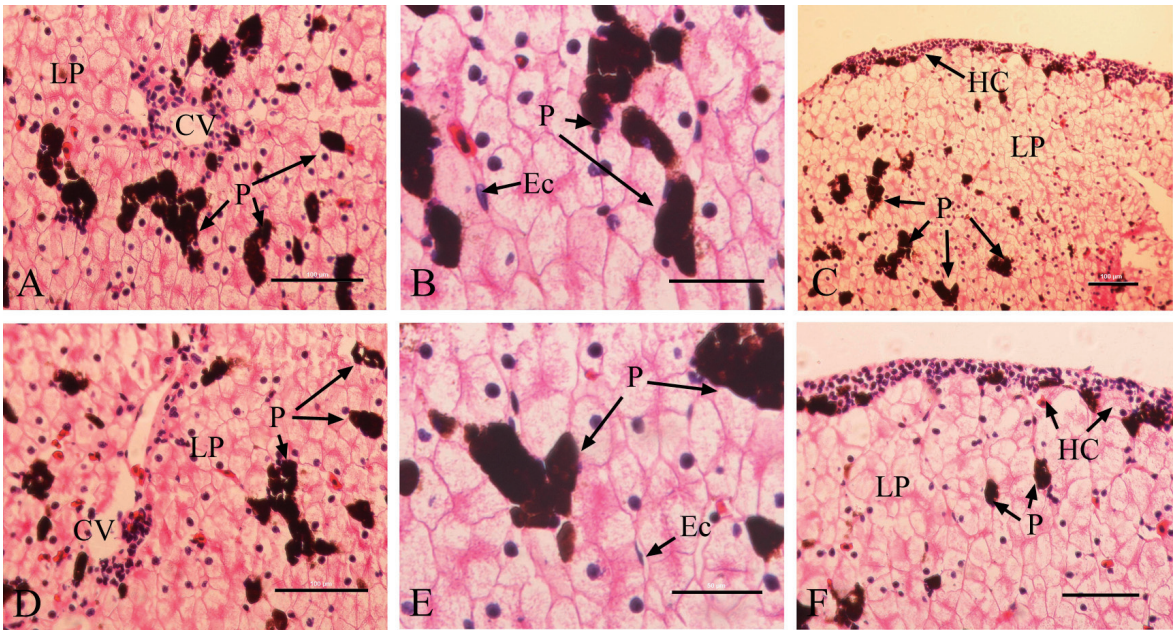

Figure 1 Distribution of the clusters of pigment cells in the liver of *C. orientalis* during hibernation (A, B, C) and activity periods (D, E, F). P: Pigmentation; LP: Liver parenchyma; HC: Haematopoietic subcapsular tissue; CV: Central vein; Ec: Endothelial cell of sinusoid. Bars: A, C, D, F = 100 μm; B, E = 50 μm.

cycle are shown in Table 1. Differences in the amount and distribution of the liver pigmentation quantified by the image analysis technique were found between the activity and hibernation periods. The amount of the pigmentation significantly increased in the hibernation period than in the activity period ($P < 0.05$). Compared with 16% during the hibernation period in winter, the pigmentation area accounted for 9% of the hepatic parenchyma during the activity period in summer. Moreover, it is worthwhile to note that the liver is heaver in winter than in summer ($P < 0.05$), and the diameters of the cell and nucleus, and the V_V (V_V=$V_{nucleus}$ / V_{cell}) are much larger in activity than in hibernation period. The hepatocyte volume in the activity period are about 14.64 % larger than those in hibernation period, while the nucleus are about 7.43% bigger in the activity period compared with those in hibernation period.

4. Discussion

Morphological and morphometric analyses were performed on the pigment cells and pigmentation of the Chinese fire-bellied newt's liver during the activity and hibernation periods. The aim of this study was to investigate the distinctive morphology of the pigment cells and the changes of the pigmentation in the newt liver during an annual cycle.

The pigment cells present in the livers of different fishes, amphibians and reptiles belong to the extracutaneous pigment system (Breathnach, 1988), and in the liver of amphibians they are localized mainly in the sinusoid walls and possess autonomous melanin synthetic activity (Scalia *et al.*, 1988). The previous studies demonstrated that the Kupffer cells of frog possess an active tyrosinase gene that could significantly increase expression during the hibernation period, thus, increase dopa oxidase activity and melanin content (Cicero *et al.*, 1989; Guida *et al.*, 2000). The liver of Chinese fire-bellied newt showed the increase in accumulation of the melanin content during the hibernation period. This phenomenon can be explained as that the liver tissue under hypoxic crisis can rapidly neutralize purines resulting from lysis of the nucleated red blood cells, and synthesis of the melanin polymer is possible, through the well-known capacity of ferrous iron, to activate tyrosinase, and also the liver tissue under hypoxic crisis can promote the synthesis of melanin granules that go against endogenous or exogenous cytotoxic (Frangioni *et al.*, 2005; Gallone *et al.*, 2007). In particular, the pigmentation plays a protective role as an antioxidant biopolymer against lipid peroxidation, which could reduce lipoperoxidation in pigmented tissue (Scalia *et al.*, 1990). In conclusion, the liver pigmentation of Chinese fire-bellied newt being increased significantly during the hibernation period is, for this newt, to be adapted to the hypoxic environment, aiming to prolong survival time.

This study finds that the V_V (V_V = $V_{nucleus}$ / V_{cell})

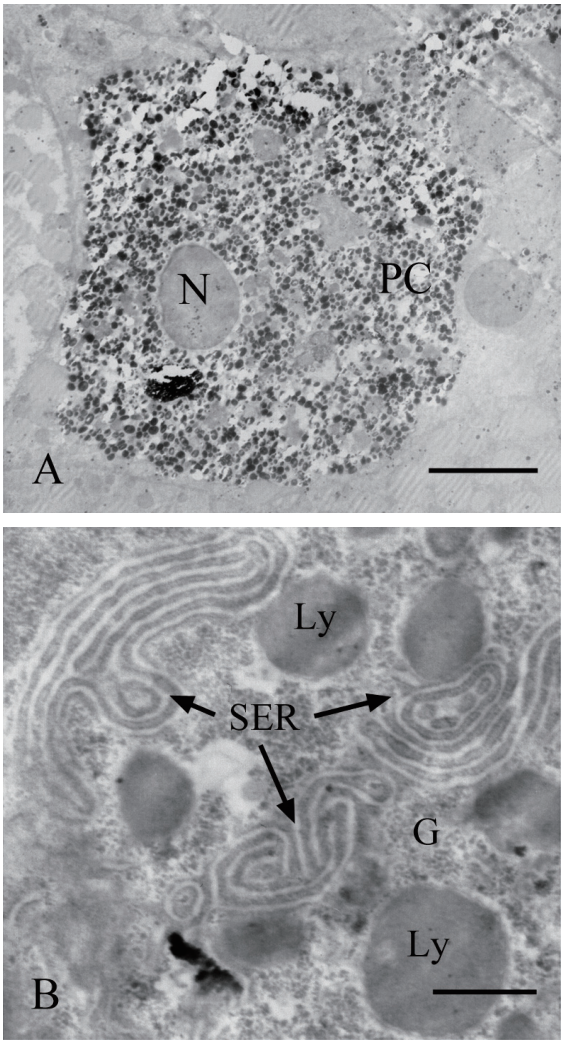

Figure 2 Ultrastructure of a large pigment cell full of melanosomes of *C. orientalis*. A: The cell being rounded by pigments in the liver; B: Patterns of cytoplasm in the pigment cell; N: Nucleolus; SER: Smooth endoplasmic reticulum; Ly: Lysosome; G: Glycogen; PC: Pigment cell. Bars: A = 10 μm; B = 1 μm.

are much larger in activity than in hibernation period. The cells in activity period are larger, about 14.64%, than those in hibernation, while the nucleus is about 7.43% bigger in activity period compared with that in hibernation period. While the previous studies found that when the frog *Rana esculenta* L. enters hibernation and is adapted to the changed environment, the frog's hepatocytes become hypertrophic, storing large amounts of materials (e. g., glycogen and lipids), and scarcely contains organelles, mainly in the segregated areas in the pre-hibernation period, followed by drastic glycogen and lipids depletion (Barni and Bernocchi, 1991; Fenoglio *et al*., 1992). The pigmentation in the liver increases during the hibernation period, but the mitochondrial SOD activity disappears, reducing the metabolic activity. After this period the SOD increases, while the pigmentation decreases in the same amount. In addition, some studies demonstrated that in the pigmented liver the antioxidant activity of the melanin could mimic part of the function of SOD (Sichel *et al.*, 1987; Sichel, 1988). We also found that the pigmentation was shown increasing in the liver of Chinese fire-bellied newt. It was correlated with functional modifications in the hepatocytes, which were characterized by a decrease in metabolic activity during this period and this kind phenomenon may be considered a kind of ecological strategy to make the liver adapted to the adverse conditions during hibernation.

Acknowledgements This work was supported by the initial funding from Henan University of Urban Construction and a grant from the Foundational and Advanced Techniques Foundation of Henan, China (122300410356). The authors are grateful to Yujie HOU for her great help in specimen collection and Junying YANG from the College of Life Sciences and Key Laboratory for Cell Differentiation Regulation, Henan Normal University for her technical assistance with electron microscope.

References

Agius C. 1979. The role of melano-macrophage centres in iron storage in normal and diseased fish. J Fish Dis, 2: 337–343

Agius C., Roberts R. J. 2003. Melano-macrophage centres and their role in fish pathology. J Fish Dis, 26(9): 499–509

Alcorn S. W., Murra A. L., Pascho R. J. 2002. Effects of rearing temperature on immune functions in sockeye salmon (*Oncorhynchus nerka*). Fish Shellfish Immunol, 12(4): 303–334

Barni S., Bernocchi G. 1991. Internalization of erythrocyte into liver parenchymal cells in naturally hibernating frogs (*Rana esculenta* L.). J Exp Zool, 258: 143–150

Barni S., Bertone V., Croce A. C., Bottiroli G., Bernini F., Gerzeli G. 1999. Increase in liver pigmentation during natural hibernation in some amphibians. J Anat, 195(1): 19–25

Barni S., Vaccarone R., Bertone V., Franschini A., Berinini F., Fenoglio C. 2002. Mechanisms of changes to the liver pigmentary component during the annual cycle (activity and hibernation) of *Rana esculenta* L. J Anat, 200(2): 185–194

Blazer V. S., Facey D. E., Fournie J. W., Coutrney L. A., Summers J. K. 1994. Macrophage aggregates as indicators of environmental stress. In Stolen J. S, Fletcher F. H. (Eds), Modulators of fish immune responses. New Jersey: SOS Publications, 169–185

Breathnach A. S. 1988. Extracutaneous melanin. Pigment Cell Res, 1(4): 234–237

Cicero R., Mallardi A., Maida I., Gallone A., Pintucci G. 1989. Melanogenesis in the pigment cells of *Rana esculenta* L. liver: Evidence for tyrosinase-like activity in the melanosome protein fraction. Pigment Cell Res, 2(2): 100–108

Fenoglio C., Bernocchi G., Barni S. 1992. Frog hepatocyte modifications induced by seasonal variations: A morphological and cytochemical study. Tissue Cell, 24: 17–29

Frangioni G., Borgioli G., Bianchi S. 2003. Melatonin, melanogenesis, and hypoxic stress in the newt, *Triturus carnifex*. J Exp Zool, 296A: 125–136

Frangioni G., Santoni M., Bianchi S., Franchi M., Fuzzi G., Marcaccini S., Varlani C., Borgioli G. 2005. Function of the hepatic melanogenesis in the newt, *Triturus carnifex*. J Exp Zool Part A: Com Exp Biol, 303(2): 123–131

Gallone A., Sagliano A., Guida G., Ito S., Wakamatsu K., Capozzi V., Perna G., Zanna P., Cicero R. 2007. The melanogenic system of the liver pigmented macrophages of *Rana esculenta* L. tyrosinase activity. Histol Histopathol, 22(10): 1065–1075

Guida G., Gallone A., Maida I., Boffoli D., Cicero R. 2000. Tyrosinase gene expression in the Kupffer cells of *Rana esculenta* L. Pigment Cell Res, 13(6): 431–435

Jordanova M., Miteva N., Rocha E. 2008. A qualitative and quantitative study of the hepatic pigmented macrophage aggregates during the breeding cycle of Ohrid trout, *Salmo letnica* Kar (Teleoestei, Salmonidae). Microsc Res Tech, 71(11): 822–830

Mizell S. 1965. Seasonal changes in energy rservesin the common frog, *Rana pipiens*. J Cell Comp Physio, 66(2): 251–258

Moura L. R., Santos A. L. Q., Belleti M. E., Vieira L. G., Orpinelli S. R. T., De Simone S. B. S. 2009. Morphological aspects of the liver of the freshwater turtle *Phrynops geoffroanus* Schweigger, 1812 (Testudines, Chelidae). Braz J Morphol Sci, 26: 129–134

Pintucci G., Manzionna M. M., Maida I., Boffi M., Boffoli D., Gallone A., Cicero R. 1990. Morpho-functional characterization of cultured pigment cells from *Rana esculenta* L. Liver. In Vitro Cell Dev Biol, 26(7): 659–664

Scalia M., Geremia E., Corsaro C., Santoro C., Baratta D., Sichel G. 1990. Lipid peroxidation in pigmented and unpigmented liver tissues: Protective role of melanin. Pigment Cell Res, 3(2): 115–119

Scalia M., Geremia E., Corsaro C., Santoro C., Sciuto. S., Sichel G. 1988. The extracutaneous pigmentary system: Evidence for the melanosynthesis in Amphibia and Reptilia liver. Comp Biochem Physio, 89(4): 715–717

Sichel G. 1988. Biosynthesis and function of melanins in hepatic pigmentary system. Pigment Cell Res, 1(4): 250–258

Sichel G., Corsaro C., Scalia M., Sciuto S., Geremia E. 1987. Relationship between melanin content and superoxide dismutase (SOD) activity in the liver of various species of animals. Cell Biochem Funct. 5(2): 123–128

Slominski A., Tobin D. J., Shibahara S., Wortsman J. 2004. Melanin pigmentation in mammalian skin and its hormonal regulation. Physiol Rev, 84(4): 1155–1228

Van Furth R., Cohn Z. A., Hirsch J. G., Humphrey J. H., Spector W. G., Langevoort H. L. 1972. The mononuclear phagocyte system: A new classification of macrophages, monocytes, and their precursor cells. Bull World Health Organ, 46(6): 845–852

Zuasti A., Jara J. R., Fetter C., Solano F. 1989. Occurrence of melanin granules and melanosynthesis in the kidney of *Sparus auratus*. Pigment Cell Res, 2(2): 93–99